1

COMPOUNDS BINDING TO P-SELECTIN

The present invention relates to compounds which bind selectively to the adhesion molecule human P-selectin, to methods for preparing such compounds, to the use of such compounds in therapeutic or diagnostic methods and in pharmaceutical compositions, to binding molecules binding to said compounds, and to a method for determining whether a compound is capable of binding to P-selectin.

BACKGROUND OF THE INVENTION

In recent years, cell surface adhesion molecules have become recognised as key mediators in numerous cellular processes including cell growth, differentiation, immune cell transmigration and response and cancer metastasis.

Four major categories of adhesion molecules have been identified: the immunoglobulin superfamily cell adhesion molecules (CAMs), cadherins, integrins and selectins.

The selectins represent a family of presently three transmembraneous, carbohydrate-binding glycoproteins: "endothelial" E-selectin, "leukocyte" L-selectin and "platelet" P-selectin. All three selectins are divalent cation (e.g. calcium) dependent and possess an extracellular domain with a carbohydrate recognition motif, an epidermal growth factor-like motif, and some smaller domains related to complement-regulatory proteins.

Human P-selectin (also referred to as GMP-140, LECAM-3, PADGEM, CD62 and CD62P) is expressed by platelets and endothelial cells. When expressed on the surfaces of these cells, its most notable effect is the slowing of leukocytes as these leave the capillaries and enter the postcapillary venules, the latter representing the major site of leukocyte-endothelium adhesion. The slowing process is observed as leukocyte rolling, signifying an initial adhesion with relatively low affinity. The firm adhesion of rolling leukocytes is primarily mediated by integrins.

In endothelial cells, P-selectin is stored on Weibel-Palade bodies; in platelets, it is found in the α-granules. Following activation, P-selectin is mobilised to the cell surfaces within a few minutes in response to a variety of inflammatory or thrombogenic agents. The endothelial P-selectin's primarily function is to recruit leukocytes into postcapillary venules, while platelet P-selectin also results in the formation of thrombi. One of the presently known

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2

natural ligands of P-selectin is PSGL-1 (P-selectin glycoprotein ligand-1), a 120 kDa

sialoprotein expressed on the surface of leukocytes where it is concentrated at the uropod.

More detailed descriptions of the structure and functions of P-selectin are found in numerous

publications (e.g. J. Panes, Pathophysiology 5: 271 (1999); F. Chamoun *et al.*, Frontiers in

5   Bioscience 5: e103 (Nov. 1, 2000) and S.-I. Hayachi, Circulation 102: 1710 (2000)).

Inflammation and inflammatory processes play a major role in the pathophysiology of

numerous diseases and conditions. Conditions of the brain in which increased selectin levels

were found and which may therefore involve selectin-mediated pathophysiological events,

include severe traumatic brain injury, relapsing-remitting multiple sclerosis, cerebral artery

10   occlusion, ischemia and stroke. Conditions of the heart in which selectins are suggested to

play a role include acute myocardial infarct, arterial injury, such as produced by angioplasty,

and ischemia. Similarly, selectins are involved in conditions of the kidneys, such as renal

injury from ischemia and reperfusion, and renal failure. Furthermore, selectins appear to play

a role in organ transplant rejection, cold ischemia, hemorrhagic shock, septic shock, tumour

15   metastasis, chronic inflammation, rheumatoid arthritis, inflammatory bowel disease,

atherosclerosis, restenosis, angiogenesis, disseminated intravascular coagulation, adult

respiratory stress syndrome and circulatory shock.

Thus, it would seem feasible to improve these and other conditions involving the

activation of endothelial cells and leukocytes and specifically the mobilisation and

20   expression of P-selectin by specifically interrupting the P-selectin cascades. This can be

done, for instance, by the administration of ligands which selectively bind to human P-

selectin, but which do not possess its bioactivity. By this method, mobilised P-selectin could

be inactivated and leukocyte-induced tissue damage prevented. Potentially, the same effect

could be achieved by gene therapy, provided the P-selectin ligand or antagonist is a peptide

25   or modified peptide. According to this method, somatic cells of a person in need of the

therapy would be transfected with an expression vector carrying a DNA sequence encoding

a P-selectin antagonist.

P-selectin ligands or antagonists may also be used for the prevention of diseases and

conditions described above. Furthermore, such ligands may also be useful in the *in vivo* or *in*

30   *vitro* diagnosis of these diseases.

Various attempts have been made in recent years to identify or create such selective

ligands to P-selectin. So far, a number of substances were tested, but no clinical studies have

yet provided conclusive evidence that any of these compounds produce the desired clinical effects while being tolerable in terms of side effects.

For instance, antibodies to P-selectin that were produced and tested in animal models, were found to protect kidneys from ischemic-reperfusion injury (H.C. Rabb *et al.*, JASN 5:

5   907 (1997) and US-A-6,033,667). In another study, a recombinant soluble form of P-selectin glycoprotein ligand-1 (rPSGL-Ig) was used to inhibit thrombosis in cats (M.J. Eppihimer *et al.*, Arteriosclerosis, Thrombosis, and Vascular Biology 20: 2483 (2000)). WO-96/09309 discloses oligosaccharide structures that are ligands to E- and P-selectin. WO-99/41363 discloses podocalyxin-like proteins that bind to selectins. WO-00/41711 describes various

10   smaller peptides or peptide sequences that bind to members of the human selectin family; most of the sequences comprise one or more units of leucine or isoleucine.

As another approach to inhibit the P-selectin cascade, various peptides derived from the lectin domain of the selectin family were found to inhibit neutrophil adhesion to P-selectin (e. g. US-A-6,111,065 and US-A-5,916,876); these peptides probably bind to P-

15   selectin receptors on leukocytes.

In WO-94/05269, peptides are described which inhibit binding of selectins such as P-selectin, E-selectin and L-selectin. These peptides have as their core region portions of the 11-18 amino acid sequence of P-selectin, E-selectin or L-selectin. Further, WO-95/31210 relates to peptides and compounds that bind selectins including endothelium leukocyte

20   adhesion molecule 1 (ELAM-1). These peptides are used for blocking adhesion of leukocytes to the selectins, *i.e.* especially E-selectin, but also P-selectin or L-selectin, for the purpose of inhibiting inflammation.

In not pre-published European patent application No. 01203314.8 compounds, which comprise the peptidic sequence $XA_xA_3A_1A_2A_1Y$ or a functional equivalent thereof and have

25   selective affinity to human P-selectin, have been disclosed wherein:

A_1 is a D- or L-cysteine (C), D- or L-methionine (M), D- or L-valine (V) or an analogue thereof;

A_2 is a D- or L- aspartic acid (D) or an analogue thereof;

A_3 is a D- or L- phenylalanine (F), D- or L-tyrosine (Y) or D- or L-tryptophan (W) or an

30   analogue thereof;

A_x is a D- or L-amino acid;

X marks the N-terminal side of said sequence and

Y marks the C-terminal side of said sequence or

4

X and Y together can form a cyclic system.

The compound EWVDV described in the above-mentioned application has an affinity for P-selectin, as expressed by an IC_{50}–value of about 2 μM. Its tetramer on streptavidin has an IC_{50} of about 2 μM.

5   Despite the above-mentioned efforts, there is still a need for substances with selective affinity to P-selectin, which can be used for preparing pharmaceutical compositions for the diagnosis, prevention and treatment of various diseases and conditions involving the adherence of leukocytes to vascular endothelial cells or to platelets. For *in vivo* use it would be highly desirable to have relatively simple compounds with a very high affinity for P-

10   selectin. There is also a need for P-selectin ligands, which can be used as targeting molecules or moieties in pharmaceutical compositions for the targeting of drugs or genetic material to tissues expressing P-selectin.

SUMMARY OF THE INVENTION

15   It is therefore an object of the invention to provide compounds with selective affinity to human P-selectin.

In particular, it is an object of the invention to provide compounds which act as antagonists or partial antagonists of P-selectin.

It is another object of the invention to provide compounds which act as targeting

20   ligands with an ability to target drugs and genetic material to cells and tissues expressing P-selectin.

A further object of the invention is the provision of methods for preparing such compounds.

Yet another object is the presentation of uses of such compounds, and of compositions

25   which contain the compounds.

LEGENDS TO THE FIGURES

Figure 1 shows the chemical structures of acyl moieties 1-7 introduced to core sequence compound 8.

30   Figure 2 is a schematic representation of the solid phase synthesis of compound library 10. Core sequence compound 8 was synthesised using standard Fmoc chemistry on Tentagel S-NH$_2$ resin with HMPA linker. The core peptide was then derivatised at the N-terminus (after removal of the Fmoc) or on the C-terminus (after selective removal of the DDE group

by 2% hydrazine) with 7 different carboxylic acids (see figure 1). The peptides were liberated from the resin by TFA cleavage resulting in compound library 10. *Fmoc,* N-(9-fluoroenyl)methoxy-carbonyl; *HMPA,* 4-hydroxymethylphenoxyacetic acid; *Boc,* N-(t-butoxycarbonyl); *tBu,* tert butyl; *TFA,* trifluoro acetic acid; *DDE,* 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl.

Figure 3 is a graphic representation of the ability of derivatised peptides HP*10*-HP*77* to inhibit ligand binding to human P-selectin. Crude derivatised peptides were tested in a competition assay for their ability to displace binding of TM11-PO to human P-selectin. Each bar represents the binding of TM11-PO in the presence of derivatised peptide (5 μM, n=3-5). Peptides are coded HP*ij* at which *i* and *j* refer to the acyl moiety 1-7, as shown in Figure 1 and attached to the N- and C-terminus, respectively. N/C-Unmodified amino groups are indicated by *0*.

Figures 4A-D show the chemical structure (figure 4A) and biological activity of compound library 14 (figures B-D). Crude peptide library 14 (1 μM) was tested in a competition assay of TM11-PO binding to human P-selectin. Three different spacers between the acyl moiety and the WVDV core sequence were used (R_1): no spacer (white bars), a glycyl spacer (grey bars) and amino butyric spacer (black bars). The chemical structure of the introduced acyl modification (R_2) is depicted below these bars.

Figure 5 shows a graphical representation of the competition of biotin-PAA-Le^a-SO$_3$ binding to human P-selectin ($\blacksquare$), mouse P-selectin ($\square$), human L-selectin ($\blacktriangle$) and human E-selectin ($\blacktriangledown$) by peptide 28. Wells were coated with each selectin (0.3 μg/ ml) and incubated with 0.33 μg/ ml biotin-PAA-Le^a-SO$_3$ with or without peptide 28 as described in example 3.

Figure 6 is a graphical representation of the competition of HL-60 cell binding to CHO-P cells by peptide 28 ($\blacksquare$) or EWVDV ($\bullet$). CHO-P cells seeded in 96 wells were incubated with calcein labeled HL-60 cells in the presence of peptide 28 or EWVDV. Data points are means of 12 data points in quadruplicate.

DETAILED DESCRIPTION OF THE INVENTION

The compounds of the present invention have selective affinity to human P-selectin and are derivatives of peptides or functional equivalents thereof, such as modified peptides, peptide analogues or peptidomimetics. The derivatives are represented by X(A$_x$)$_m$A$_3$A$_1$A$_2$A$_1$Y, wherein:

6

- A_1 is a D- or L-amino acid selected from the group consisting of cysteine (C) and valine (V), or an analogue or mimetic thereof;

- A_2 is a D- or L- aspartic acid (D) or an analogue or mimetic thereof;

- A_3 is a D- or L-amino acid selected from the group consisting of phenylalanine (F) and tryptophan (W), or an analogue or mimetic thereof;

- A_x is any D- or L-amino acid selected from the group consisting of glutamic acid, aspartic acid, glycine, cysteine and analogues or mimetics thereof;

- X is an N-terminal group or sequence and is hydrogen or a residue comprising 1 to 6 D- or L-amino acids or analogues thereof;

- Y is a C-terminal group or sequence and is –OH or a residue comprising 1 to 11 D- or L-amino acids or analogues thereof;

and are characterised in that either X or Y, or both X and Y, are substituted with the group:

- R^1-$(Z)_n$- wherein:

- Z is selected from the group consisting of -CO-, -O-, -NR^2-, and -CO-NR^2-;

- R^1 and R^2 are independently selected from:

- H;

- a (C_1-C_8)alkyl group;

- a (C_2-C_8)alkyl group in which at least one hydrogen substituted C-atom is replaced with a hydrogen substituted N-, oxygen- or sulphur atom;

- a (C_6-C_{14})aryl group which may be substituted with at least one group selected from the group consisting of a halogen atom, (C_1-C_6)alkyl, -CF_3, -OH, -O-(C_1-C_6)alkyl, -COOH, -COO-(C_1-C_6)alkyl, -NO_2, -NH_2, -NH-(C_1-C_6)alkyl, -N-$((C_1-C_6)$alkyl$)_2$ and -SO_3H;

- a heteroaryl group which is selected from 5- or 6-membered ring systems and benzo-condensed ring systems, and has at least one heteroatom selected from the group consisting of nitrogen, oxygen and sulphur, wherein said heteroaryl group may be substituted with at least one group selected from the group consisting of a halogen, -(C_1-C_6)alkyl, -CF_3, -OH, -O-(C_1-C_6)alkyl, -COOH, -COO-(C_1-C_6)alkyl, -NO_2, -NH_2, -NH-(C_1-C_6)alkyl, -N-$((C_1-C_6)$alkyl$)_2$ and -SO_3H; or

- an aralkyl group comprising an alkyl group as defined above and an aryl group or heteroaryl group as defined above.

The indices m and n represent integers independently selected from 0 and 1. However, n is not 0 when R^1 is H.

Furthermore, a compound of the invention comprises a derivative of a peptide or a molecular structure that is related to a peptide, herein referred to as functional equivalent.

Peptides are defined as amides that are derived from two or more amino acids by combination of the amino group of one acid with the carboxyl group of another (Merriam

5   Webster Medical Dictionary ©2001). As used herein, a peptide may also refer to a peptidic structure within a molecule. Typically, peptides are composed of naturally occurring L-α-amino acids, which are alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine

10   (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V).

Functional equivalents of the peptides of the invention are proteinaceous molecules, comprising the same human P-selectin binding activity in kind, but not necessarily in

15   amount, and may, for instance, be modified peptides, peptoids, peptide analogues or peptidomimetics.

Modified peptides are molecules derived from peptides by the introduction of substituents or functional groups which are not present in naturally occurring amino acids. The term also includes compounds which are obtained by the reaction of peptides with

20   molecules from other chemical categories, whether these molecules are naturally occurring or not. For instance, biotinylated peptides, glycoproteins, and lipoproteins are frequently found in nature, while peptides modified with polyethylene glycol, such as pegylated interferon α-2b (Peg-Intron®), are examples of chemically modified peptides that have been designed to alter some, but not all of the peptides' properties.

25   Peptoids, like peptides, are typically amides of two or more amino acids. However, they are frequently not directly derived from naturally occurring amino acids, but rather of various types of chemically synthesised L- or D-amino acids.
Peptidomimetics, in their broadest scope, are compounds which are in their functional structure more or less similar to a peptide, but which may also contain non-peptidic bonds in

30   the backbone, or D-amino acids. In general, peptidomimetics serve as substitutes for native peptides in the interaction with receptors and enzymes (Pharmaceutical Biotechnology, Ed. D.J.A. Crommelin and R.D. Sindelar, Harwood Academic Publishers, 1997, p. 138).

Pseudopeptides, a class of peptidomimetics, are compounds containing amide bond isosteres instead of amide bonds (*ibid.*, pp. 137-140).

According to the invention, the two units of A_1 within the sequence are selected independently; they can be identical or different from each other. Preferably, at least one of

5   the A_1 units represents valine (V). More preferably, both A_1 units are valine (V). In another embodiment, A_1 is an analogue or mimetic of cysteine (C), methionine (M), or valine (V). Techniques for selecting appropriate analogues or mimetics of naturally occurring amino acids are well-known to those skilled in the art. For example, the pseudopeptide approach aims at achieving a higher chemical or enzymatic stability of a peptide while retaining its

10   bioactivity. Peptide bonds which undergo rapid degradation *in vivo* are replaced by other functional groups to create bioisosteres or amide bond surrogates. Examples of bioisosteric groups are N-methyl amide, thioester, thioamide, ketomethylene, methyleneamino, retro-inverse amide, methylenethio, and methyleneoxy groups (*ibid.*, pp. 138-139). An approach to improve the selectivity of a peptide is to introduce conformational constraints to the

15   backbone, which decreases the number of potential receptor or enzyme interactions. The constraints are most often achieved by the introduction of carbon-carbon double bonds (olefinic analogues) and ring structures (*ibid.*). A further method to identify analogues which mimic the functional structure of an amino acid such as cysteine (C), methionine (M), or valine (V) is computer-aided modeling. Preferred mimetic structures possess a side chain

20   with similar charge or electronegativity, hydrophobicity and spatial orientation to said amino acids. In another embodiment, one or both A_1 units are D-amino acid analogues of cysteine (C), methionine (M), or valine (V).

As said, the present invention also encompasses derivatised functional equivalents of the said peptides. Equivalents can, among others, be found by amino acid substitution using

25   conservative amino acid changes. Particularly, the invention also encompasses a method for determining whether a compound is capable of binding to human P-selectin, comprising substituting in a compound according to the invention, an amino acid for a conservative amino acid and determining whether the resulting compound is capable of binding to said P-selectin. According to the invention, A_2 is a D- or L-aspartic acid with a solvent exposed

30   side chain bearing a negative charge at physiological pH, or an analogue or mimetic thereof. For identifying appropriate analogues, the same principles apply as those that have been set forth with regard to A_1.

A_3 is a D- or L-amino acid with an aromatic side chain selected from the group

consisting of phenylalanine (F), and tryptophan (W), or an analogue or mimetic thereof.

More preferably, A_3 is an L-amino acid selected from this group. Most preferred is

tryptophan (W). In other embodiments, A_3 is an analogue structure related to phenylalanine

5   (F), or tryptophan (W) as defined above.

A_x is a D- or L-amino acid, as defined above, or an analogue or mimetic thereof. In

one of the preferred embodiments, A_x is a D- or L- glutamic acid (E), or an analogue or

mimetic thereof.

It has to be noted that the 2 units of A_1 are selected independently,

10   i. e. they can be identical or different from each other.

X marks the N-terminal group. X can simply be a hydrogen. In another embodiment,

X is a sequence of up to 6 amino acids, or analogues or mimetics thereof. The type of

amino acids(s) should of course not affect e.g. by changing the configuration of the

peptide or by changing the spatial orientation of the terminal carboxylic acid with respect

15   to the core peptide the recognition by P-selectin in such a way that, the aimed effect is no

longer obtained. Suitable analogues include amino substituted carboxylic acids, e.g.

aminocyclohexanoic acid amino benzoic acid and amino caproic acid.

Y marks the C-terminal group. Y may be a hydroxyl group. In a preferred

embodiment, Y is a residue comprising 1 to 11 amino acids carrying a terminal hydroxyl

20   group. The recognition by P-selectin is more tolerant to substituents at the C-terminus. It is

also preferred that at least one amino acid with a negatively charged side chain is present in

Y, preferably being separated from the remaining $X(A_x)_m A_3 A_1 A_2 A_1$ sequence by one to

three amino acids. In a particularly preferred embodiment of the invention Y is D- or L-

lysine (K), or comprises D- or L- lysine (K).

25   The compound of the invention can possess a cyclic or otherwise constrained

backbone. In that case, X+Y together may suitably be linked through an S-S-binding,

although, of course, also other types of (chemical) linkages can be present, such as an amide

binding, a thioether binding, a carbamate binding or an ester binding.

The length of the group X+Y is not particularly critical as long as the core sequence is

30   recognised by P-selectin. Generally, the minimum length of X+Y corresponds with the

length of 5-6 amino acids.

The group R^1-$(Z)_n$- with which X or Y, or both X and Y independently, are

substituted, may be selected from R-CO groups wherein R is an alkyl, aryl or heteroaryl

group. The alkyl group preferably has 2 to 8 carbon atoms, at least one of which may be replaced by a nitrogen, oxygen, or sulphur moiety. An aryl group has preferably 6 to 14 carbon atoms, and the group may be substituted with at least one group selected from the group consisting of OH, COOH, NO_2, NH_2, SO_3H and CF_3. Also substitutions on this group selected from $-NHR$, $-CO(NHR)$, $-CN(NHR)$, $-N_3$, $-(CH_2)_p-OH$, and $-(CH_2)_p-COOH$ are suitable, wherein R is as defined hereinabove, while p is an integer of 1-8. The heteroaryl group has the same definition as the aryl group with the exception that at least one heteroatom selected from the group consisting of nitrogen, oxygen and sulphur is incorporated instead of a carbon atom. Preferably, the heteroaryl group is based on a 5- or 6-membered ring system or benzo-condensed ring system.

Examples of compounds are those wherein Z is -CO-, and R^1 and/or R^2 represent independently from each other the following groups: 4-hydroxyphenylcarbonyl, 3,5-dihydroxyphenylcarbonyl, 3-hydroxy-5-carbonyloxyphenylcarbonyl, 3,4,5-tri-methoxy-phenylcarbonyl, 3,5-di-nitro-phenylcarbonyl, 4-carboxyphenylcarbonyl, 3-carbonylbutyric acid, 2-hydroxy-5-sulfonylphenylcarbonyl, 3-carboxyphenylcarbonyl, 3,5-difluorophenylcarbonyl, 1-sulfonyl-2-aminoethylcarbonyl, 3,5-di(trifluoromethyl)phenylcarbonyl, hydroxymethylcarbonyl and 4-nitrophenylcarbonyl. However, especially preferred groups are acyl groups and more particularly 3,5-dicarboxyphenylcarbonyl and 3,4,5-trihydroxyphenylcarbonyl groups.

Further, also modifications based on tannic acid, caffeinic acid, oligohydroxyl aryl groups and oligocarboxy-aryl groups as well as derivatives thereof are preferred, especially on group X. The 3,4,5-trihydroxyphenylcarbonyl group however can be used advantageously both on group X and group Y.

It is assumed that the R^1-$(Z)_n$ modification confers additional interaction of the peptide compound with the P-selectin binding pocket on P-selection, possibly by electrostatic interaction or H-bridge formation. This leads to gain inaffinity for P-selectin.

Very good results are obtained for those compounds having an N-terminal amide function. Also very good results are obtained when using a linker between the N-terminal substituent group and amino acid A_x or A_3 in the amino acid sequence of the compounds of the invention. Suitable linkers or spacers are glycine and aminobutyric acid.

Compounds of the invention possess linear, branched, cyclic, or constrained backbones. For instance, peptides or functional equivalents with at least two cysteine (C) units can be cyclised by oxidation. If a compound is cyclised via such a disulfide bond, it

11

is preferred that the participating cysteine units are members of X and Y, respectively. Cyclic structures are, in fact, an example for conformationally constrained backbones. Other types of constrained structures may also be introduced to decrease the conformational flexibility of the compound. Especially the presence of olefinic bonds or small ring structures in the backbone serves this purpose. Examples of such constraints are given in Pharmaceutical Biotechnology, Ed. D.J.A. Crommelin and R.D. Sindelar, Harwood Academic Publishers, 1997, p. 139f.

In one of the preferred embodiments, the compounds of the invention are provided as multimers of the derivatised peptides or functional equivalents thereof.

In another type of multimer that can be created to form the compounds of the invention, single derivatised peptide or peptoid chains in accordance with the invention are coupled to a biocompatible protein, such as human serum albumin, humanised antibodies, liposomes, micelles, synthetic polymers, nanoparticles, and phages. Multimers can also represent derivatised peptides which are serially coupled to each other via spacers, i.e. concatamers, or dendrimers, or clusters.

The compounds can generally be prepared by the methods that are known for the preparation of peptides and similar substances and derivatives thereof. Smaller compounds containing only a few amino acids or similar units, and preferably not more than 30-50 units, can be prepared by chemical and/or enzymatic ligation techniques, either using the classical approach in which the reactions take place in solution or suspension, or by employing the more modern solid phase approach, in which the peptide is assembled while being anchored to a solid surface, such as a polymeric bead. Larger compounds are typically synthesised by automatic solid phase peptide synthesisers. The compounds obtained are then modified by reaction with a suitable $R^1(Z)_n$- source, preferably a $R^1(Z)_n$- OH group. Said source reacts with a primary amine group in the peptide compound.

When the peptide sequence in the compounds according to the invention is made by standard Fmoc chemistry and the protecting Fmoc group is removed, the N-terminus is immediately available for the reaction introducing the $R^1(Z)_n$-group.

When the C-terminus has no free amine available, the said source can be reacted with an added lysine, which lysine can suitably be added as its DDE derivative. DDE is 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethyl; it can selectively be removed with 2% hydrazine without affecting acid-labile side chain protecting groups.

12

Some more comprehensive summaries of methods which can be applied in the preparation of the compounds are described in: W.F. Anderson, Nature 392 Supp., 30 April 1998, p. 25-30; Pharmaceutical Biotechnology, Ed. D.J.A. Crommelin and R.D. Sindelar, Harwood Academic Publishers, 1997, p. 53-70, 167-180, 123-152, 8-20; Protein Synthesis:

5　Methods and Protocols, Ed. R. Martin, Humana Press, 1998, p. 1-442; Solid-Phase Peptide Synthesis, Ed. G.B. Fields, Academic Press, 1997, p. 1-780; Amino Acid and Peptide Synthesis, Oxford University Press, 1997, p. 1-89.

Salts of the derivatives of the peptides or functional equivalents thereof are prepared by known methods, which typically involve the mixing of the derivative of the peptide or

10　peptoid with either a pharmaceutically acceptable acid to form an acid addition salt, or with a pharmaceutically acceptable base to form a base addition salt. Depending on the intended use pharmaceutically acceptable acids include organic and inorganic acids, such as formic acid, acetic acid, propionic acid, lactic acid, glycolic acid, oxalic acid, pyruvic acid, succinic acid, maleic acid, malonic acid, cinnamic acid, sulfuric acid, hydrochloric acid, hydrobromic

15　acid, nitric acid, perchloric acid, phosphoric acid and thiocyanic acid, which form ammonium salts with free amino groups of the derivatised peptides and functional equivalents thereof. Pharmaceutically acceptable bases, which form carboxylate salts with free carboxylic groups of the derivatised peptides and functional equivalents thereof, include ethylamine, methylamine, dimethylamine, triethylamine, isopropylamine, diisopropylamine,

20　and other mono-, di- and trialkylamines, as well as arylamines.

Moreover, also pharmaceutically acceptable solvates are encompassed.

Multimers of the derivatised peptide or peptoid according to the invention can, for example, be prepared in a similar way as multimers of peptides, e.g. by biotinylating the N-terminus chains and subsequent complexation with streptavidin, thereby if deemed

25　necessary adjusting the method to the specific compounds. As streptavidin is able to bind 4 biotin molecules or conjugates with high affinity, very stable tetrameric peptide complexes can be formed by this method. Multimers may be composed of identical or different derivitised peptides or functional equivalents. Preferably, however, the multimers of the invention are composed of two or more identical derivatised peptides or functional

30　equivalents thereof.

The compounds of the invention have an affinity to human P-selectin, a membrane glycoprotein expressed by vascular endothelial cell and platelets, which is involved in leukocyte adhesion to the endothelium and platelets. The affinity or binding characteristics

of compounds to P-selectin can be quantified, for example, in terms of the IC_{50}–value. Typically, an IC_{50}–value of about 50-100 µM or less would be considered as evidence for affinity and binding. More desirable for ligands are substances with IC_{50}–values of about 10 µM or less. The lowest a IC_{50}–value attainable for the non-covalent type bonds playing a

5   role in the interactions or bindings in accordance with the present invention is about 10^{-15} M. Generally, however, the IC_{50}–values are higher than about 10^{-12} M and in most cases higher than about 10^{-9} M.

The compounds of the invention have low micromolar to low nanomolar IC_{50}-values towards P-selectin, which make them about equally potent as the natural ligand P-selectin

10   glycoprotein ligand-1.

It was found that N-terminally modified compounds of the invention were more effective (potent and/or specific) than the corresponding C-terminally modified compounds containing the same modifications. Very good results were obtained with modification by 1,3,5-tricarboxylic acid or gallic acid.

15   In addition thereto the compounds according to the invention may render the peptides more stable against proteases, particularly against N-exopeptidases, due to the derivatisation. The compounds further may have an enhanced specificity for P-selectin and therefore modulate a pharmacological action such as interaction between monocyte and platelet, that between monocyte and endothelial cells, that between platelet cells and platelet cells.

20   Potential side effects may thus also be diminished. The compounds would be cross-reactive in many species.

A further aspect of the invention refers to the uses of the disclosed compounds. Since the compounds bind selectively to P-selectin, they can, depending on their type of interaction with P-selectin after binding, function as antagonists, partial antagonists, or as mere

25   targeting means to target conjugated substances to cells and tissues expressing P-selectin. Thus, the compounds can be advantageously used in pharmaceutical compositions.

Pharmaceutical compositions containing the compounds of the invention may be adapted for various routes of administration, such as parenteral, oral, transmucosal, nasal, or pulmonary. They may further contain drug targeting agents, bioavailability enhancement

30   agents, or active ingredients other than compounds of the invention, and provide for immediate or modified release.

As used herein, the term "pharmaceutical composition" refers to therapeutic and diagnostic compositions, as well as to medicaments and diagnostics containing such

14

compositions. Therapeutic compositions and medicaments are used for the prevention or treatment of diseases and other conditions of mammals of which conditions improvement is desirable. Diagnostics and diagnostic compositions are used for the diagnosis of such diseases *in vivo* and *in vitro*.

5   A preferred use of the compounds is for preparing therapeutic compositions or medicaments to prevent or improve diseases and conditions involving the adhesion of leukocytes, such as monocytes and neutrophils, to the vascular endothelium and to platelets. The compounds can also be used in compositions for treating diseases in which the inhibition of P-selectin-mediated intracellular signaling is desirable.

10   For instance, compositions containing one or more compounds of the invention can contribute to controlling leukocyte-mediated inflammatory processes. It is known that activated leukocytes release toxic molecules which can damage normal tissue. These inflammatory responses, some of which also involve P-selectin-mediated platelet activation, are part of several pathological conditions, such as transplant rejection, cold ischemia,

15   hemorrhagic shock, septic shock, tumour metastasis, thrombosis, chronic inflammation, rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, restenosis, angiogenesis, disseminated intravascular coagulation, adult respiratory stress syndrome, circulatory shock, severe traumatic brain injury, relapsing-remitting multiple sclerosis, cerebral artery occlusion, ischemia, stroke, acute myocardial infarct, deep vein thrombosis, arterial injury,

20   such as produced by angioplasty, myocardial ischemia, renal injury from ischemia and reperfusion, and renal failure.

          In another embodiment, the compounds are used in the preparation of diagnostic compositions or products. Such compositions can be used for *in vitro* tests to quantify P-selectin concentrations in body fluids as markers for the diseases and conditions described

25   above. They may be also used for *in vivo* diagnostic imaging procedures to monitor P-selectin mediated atherosclerosis, aneurisms, restenosis following percutaneous transluminal coronary angioplasty (post-PTCA restenosis) and other conditions selected from those in which P-selectin is mobilised. As an option for this use a compound according to the invention may be conjugated with a chelator, which is subsequently complexed with an

30   isotropic label that is detectable by the chosen monitoring system.

          Another use of the compounds is as that of a tool in research. For instance, they can be used to test the binding affinity of molecules to P-selectin or functional equivalents of P-selectin. To conduct this test method, P-selectin or a functional equivalent of P-selectin

would be contacted and incubated with a molecule to be tested for binding affinity and with a compound of the invention. A reduced binding of the compound of the invention would indicate an affinity of the molecule to P-selectin.

The compounds can also be used as targeting molecules or conjugates in pharmaceutical compositions for the targeting of drugs or genetic material to tissues that express P-selectin. As conjugates, the compounds can be directly coupled with active molecules or nucleic acids that are to be delivered to such tissues. Alternatively, they can be incorporated into or anchored onto the surface of liposomes or other lipid vesicles, emulsion droplets, polymers, nano- or microparticles to obtain targeted vehicles for drugs or genetic material which is delivered to P-selectin expressing tissues.

The pharmaceutical compositions preferably contain one or more compounds with P-selectin affinity as disclosed herein and at least one carrier or excipient. As used herein, a carrier or excipient is any pharmaceutically acceptable substance or mixture of substances having no substantial pharmacological activity, which can be used as a vehicle or as an auxiliary substance to formulate a compound into dosage form which is stable and easy to administer. Examples of pharmaceutically acceptable excipients are found in the monographs of all major pharmacopoeias.

In one embodiment, the composition is formulated and processed for parenteral injection, preferably for intravascular injection, such as intravenous or intra-arterial, but also for intramuscular, subcutaneous, intralesional, intraperitoneal or other routes of parenteral administration. The same principles that govern the formulation of other drugs for these administration routes will also teach those skilled in the arts on how to prepare such compositions. For instance, one of the requirements of parenteral dosage forms is their sterility. Other requirements are described in all major pharmacopoeias, such as in USP 24, in the monograph "General Requirements for Tests and Assays. 1. Injections", p. 1775-1777. To increase the stability of a parenteral formulation, it may be necessary to provide a dried dosage form which must be reconstituted before it can be administered. An example of such a dosage form is a freeze-dried or lyophilised formulation.

It may be desirable to administer a compound of the invention as a parenteral controlled release dosage form to avoid frequent injections and to improve the effectiveness and convenience of the therapy. Various methods of preparing such depot formulations are known. Prolonged release may be provided by solid implants, nanoparticles, nanocapsules,

microparticles, microcapsules, emulsions, suspensions, oily solutions, liposomes, or similar structures.

Excipients that are particularly useful for the preparation of parenteral formulations are solvents, cosolvents and liquid or semisolid carriers, such as sterile water, ethanol, glycerol,
5 propylene glycol, polyethylene glycol, butanediol, fatty oils, short- and medium chain triglycerides, lecithin, polyoxyethylene castor oil derivatives; substances to adjust the osmolality and pH, such as sugars, especially glucose, sugar alcohols, especially mannitol, sodium chloride, sodium carbonate, citric acid, acetate, phosphate, phosphoric acid, hydrochloric acid, sodium hydroxide etc.; stabilizers, antioxidants, and preservatives, such
10 as ascorbic acid, sodium sulfite or -hydrogen sulfite, EDTA, benzyl alcohol etc.; other excipients and lyophilization aids, such as albumin, dextran etc.

Alternatively, the pharmaceutical compositions may be designed for oral administration and processed accordingly. Appropriate oral dosage forms include tablets, hard capsules, soft capsules, powders, granules, orally disintegrating dosage forms, syrups,
15 drops, suspensions, effervescent tablets, chewable tablets, oral films, lyophilised dosage forms, sustained release dosage forms and controlled release dosage forms. In one of the preferred embodiments, the oral dosage form is an enterically coated solid dosage form to provide protection of the compound from the acidic and proteolytic environment of the stomach.

20 It may also be advantageous to administer a compound of the invention in a transmucosal dosage form. This route of administration is non-invasive and patient-friendly; at the same time it may lead to an improved bioavailability of the compound compared to oral administration, especially if the compound is not stable in the fluids of the digestive system, or if it is too large to be absorbed from the gut effectively. Transmucosal
25 administration is possible via, for instance, nasal, buccal, sublingual, gingival or vaginal dosage forms. These dosage forms can be prepared by known techniques; they can be formulated to represent nasal drops or sprays, inserts, films, patches, gels, ointments or tablets. Preferably, the excipients used for a transmucosal dosage form include one or more substances providing for mucoadhesion, thus prolonging the contact time of the dosage form
30 with the site of absorption and thereby potentially increasing the extent of absorption.

In a further embodiment, the compounds are administered via the pulmonary route, using a metered dose inhaler, a nebuliser, an aerosol spray, or a dry powder inhaler.

17

Appropriate formulations can be prepared by known methods and techniques. Transdermal, rectal, or ocular administration may also be feasible in some cases.

It can be advantageous to use advanced drug delivery or targeting methods to deliver a compound of the invention more effectively. For instance, if a non-parenteral route of

5   administration is chosen, an appropriate dosage form may contain a bioavailability enhancing agent, which may be any substance or mixture of substances which increases the availability of the compound. This may be achieved, for instance, by the protection of the compound from degradation, such as by an enzyme inhibitor or an antioxidant. More preferably, the enhancing agent increases the bioavailability of the compound by increasing

10   the permeability of the absorption barrier, which is typically a mucosa. Permeation enhancers can act via various mechanisms; some increase the fluidity of mucosal membranes, while others open or widen the gap junctions between mucosal cells. Still others reduce the viscosity of the mucus covering the mucosal cell layer. Among the preferred bioavailability enhancers are amphiphilic substances such as cholic acid

15   derivatives, phospholipids, ethanol, fatty acids, oleic acid, fatty acid derivatives, EDTA, carbomers, polycarbophil, and chitosan.

In a further aspect, molecules capable of binding to the compounds disclosed above are within the scope of the invention. For instance, standard hybridysation technology can be applied to prepare specific monoclonal antibodies to a compound. Other techniques are

20   available to design and prepare smaller molecules capable of binding to a compound of the invention.

The following examples further illustrate the invention, but are not intended to limit its scope to the embodiments presented therein.

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EXAMPLES

Example 1

Chemical synthesis of resin-bound core sequence compound 9

5   Core sequence compound 9 (FmocHN-Glu(OtBu)-Trp(Boc)-Val-Asp(OtBu)-Val-

Lys(DDE)-GABA-HMPA-resin; see fig. 1) was synthesised on an Applied Biosystems 9050

peptide synthesizer (Warrington, UK) using standard Fmoc chemistry. In short, Tentagel S-

NH₂ (load 0.26 mmol/g) was provided with 4-hydroxymethylphenoxyacetic acid (HMPA) as

a linker, resulting in compound 8 (see fig.1). Fmoc-GABA –OH (10 eq.) was attached to the

10   HMPA resin 8 under the agency of N,N'-dicyclohexylcarbodiimide (DCC, 5 eq.) and 4-

dimethylaminopyridine (DMAP, 0.5 eq.) All other amino acids, with acid labile side chain

protection if necessary, were attached by coupling in the presence of HOBt/TBTU/Dipea

(4/4/8 eq.). After coupling, the resin was washed with DMF, isopropanol and diethylether

and subsequently dried.

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Example 2

Synthesis of compound 10

The solid phase synthesis of the compound library 10 (see fig. 1) was performed using

a Flexchem© system (Robbins Scientific, Sunnyvale U.S.A.). After removal of the N-

20   terminal Fmoc group of compound 9 by 20% piperidine in DMF, the resin was washed

(DMF) and dried. The resin was distributed in 10 mg quantities over a solvent-resistant 48-

well filter plate. After washing with DMF, a mixture of the desired carboxylic acid (40 eq.),

BOP (20 eq.), HOBt (20 eq.) and NMM (100 eq.) was added (total volume 300 μl) and the

suspended resin was incubated for 3 hours under continuous perspiration with N₂.

25   Subsequently, the resin was washed with DMF and incubated three times for 3 minutes with

hydrazine monohydrate (2% in DMF) to remove the DDE (4,4-dimethyl-2,6-

dioxocyclonhex-1-ylidene)ethyl) group. After washing with DMF, a mixture of the second

carboxylic acid, BOP, HOBt and NMM (same amounts as described above) was added and

once again incubated for 3 hours. Derivatised peptides, which were only modified at the C-

30   terminal lysine (HP01-HP07 see hereafter), were N-boculated with di-t.-butyl-dicarbonate

((t-Boc)₂ O; 0.25 M) and Dipea (0.125 M in NMP) prior to reaction to protect the N-terminal

amine function. After removal of the solvent, peptides were cleaved off from the resin with a

mixture of trifluoro acetic acid (TFA), triisopropylsilane (TIS) and water mixture (95:2.5:2.5

v/v/v). Each sample was lyophilised and stored at -20°C until use.

Example 3

Synthesis of compounds 11-13, Compound Library 14 and compounds 27-33

Derivatised peptides 11-13, compound library 14 and derivatised peptides 27-33 were

5   prepared in the same manner as described for library 10 from resin bound core sequence

FmocHN-Trp(Boc)-Val-Asp(OtBu)-Val-HMPA-resin. Derivatised peptide 11 was

acetylated using acetic anhydride (0.25 M) and Dipea (0.125 M) in NMP.

Example 4:

10   ### Competition assay with TM11-PO

Compounds according to the invention, as prepared in examples 1, 2 and 3 and some

reference peptide compounds were assayed for its ability to inhibit TM11-PO binding to

human P-selectin. TM11-PO, a tetrameric TM11/strepPO complex, which has previously

been shown to bind with high affinity and specificity to human P-selectin (Molenaar et al.,

15   Blood 2002, 100, 3570-3577), was freshly prepared by incubating streptavidin-peroxidase

(strep-PO (Amersham Life Science, Little Chalfont, United Kingdom), 8.4 μl, 2.0 μM) and

TM11-biotin (biotin-CDVEWVDVSSLEWDLPC (synthesised by Dr. Van der Zee,

Department of Immunology, University of Utrecht, Utrecht, the Netherlands), 1.5 μl 190

mM) for 2 hours at room temperature in assay buffer (20 mM HEPES, 150 mM NaCl, 1

20   mM CaCl$_2$, pH 7.4). For competition studies, a 96 wells microtiter plate (high binding, flat

bottom, Costar, Corning, U.S.A.) was coated overnight at 4°C with 10 μg/ ml goat anti-

human IgG (Fc specific) (Sigma-Aldrich, Zwijndrecht, the Netherlands) in coating buffer

(50 mM NaHCO$_3$, pH 9.6). Subsequently, wells were washed with assay buffer and

incubated for 1 hour at 37°C with blocking buffer (3% BSA in assay buffer). After washing

25   with assay buffer, the wells were incubated for 2 hours at 37°C with the human P-selectin

IgG-Fc chimera (R&D Systems Europe Ltd., Abingdon, United Kingdom) (0.3 μg/ml).

Subsequently, wells were washed with assay buffer and incubated for 1 hour at 4°C with the

TM11-PO complex. The wells were washed six times with washing buffer (0.1% Tween 20

in assay buffer). 3,3',5,5'-Tetramethylbenzamidine (TMB)/ hydrogen peroxide (H$_2$O$_2$)

30   (Pierce, Rochford, U.S.A.) was added and wells were incubated at room temperature for 15

minutes. The reaction was halted by addition of 2 M H$_2$SO$_4$ and the absorbance was

measured at 450 nm.

For the results see the tables below and figure 3.

Table 1: Derivatised peptide and IC$_{50}$ (μM)

	HP*14*	1.02
	HP*16*	0.31
	HP*17*	0.45
5	HP*61*	0.19
	HP*62*	0.28
	HP*63*	3.40
	HP*65*	2.35
	HP*60*	1.10
10	HP*06*	6.00

The derivatised peptides are coded HP*ij*, wherein *i* and *j* refer to the acyl moieties R^1 and R^2 attached to the N- and C-terminus, respectively, of formula 10 (Fig.1). Unmodified amino group is indicated by *0*. The acyl moieties 1 to 7 are shown in Fig. 2.

The affinity of the compounds was confirmed by a cell adhesion assay in which

15   Chinese hamster ovary cells expressing human P-selectin were incubated with HL60 cells expressing PSGL-1 in the presence of titered amounts of the compounds.

Table 2: Derivatised peptides and their IC$_{50}$-values for human P-selectin as determined

20   **by competition Elisa**

Peptide	Sequence	IC$_{50}$ (μM)[a]
TM11	CDVEWVDVSSLEWDLPC	2[b]
A29	EWVDV	8[b]
A129	DWVDV	16[b]
A130	KWVDV	28[b]
A131	AWVDV	27[b]
A138	WVDV	> 1000[b]
11	Ac-WVDV	27
12	GA-WVDV	0.037
13	GA-EWVDV	0.031

[a] results are the average of at least 3 experiments at 8 different concentrations of peptide as determined in TM11-PO competition ELISA (materials and methods)
[b] from not pre-published European patent application 01203314.8.

Table 3: Gallic acid derivatized peptides, and their IC$_{50}$ for human P-selectin as determined by competition Elisa.

Peptide	Structure	IC$_{50}$ (nM)[a]
12		37.1
27		18.3
28		15.4
29		62.9
30		250
31		>1000
32		590
33		580

[a] values are the mean of at least 3 experiments at 8 different concentrations of peptide as determined in TM11-PO competition ELISA (materials and methods)

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Example 5

Competition assay with PAA-Le^a-SO₃H

To be able to test the binding of compounds according to the invention also to other members of the selectin family (i.e. E- and L-selectin), biotin-PAA-Le^a-SO$_3$H (Synthesone, Munich, Germany) instead of TM11-PO was used as ligand in the competition assay. This polyacrylamide (PAA) based polymer with attached sulfo-Lewis A groups, is an established selectin ligand.

Experimentally, the same procedure was used as in example 1 however with the following differences. After washing with assay buffer, the wells were incubated for 2 hours at 37°C with the human P-selectin IgG-Fc chimera, mouse P-selectin IgG-Fc chimera, human L-selectin IgG-Fc chimera and human E-selectin IgG-Fc chimera respectively (all from R&D Systems Europe Ltd., Abingdon, United Kingdom) (0.3 µg/ml). Subsequently the wells of the microtiter plate were incubated with biotin-PAA-Le^a-SO$_3$H (0.33 µg/ml) for 2 hours at 37°C, instead of with the TM11-PO-complex.

For the results see figure 5.

Example 6

Inhibition of dynamic interactions between HL60 cells and human P-selectin expressing Chinese hamster ovary cells

Chinese hamster ovary (CHO) cells stably transfected with human P-selectin (CHO-P) were kindly donated by Dr. Modderman (University of Amsterdam, Amsterdam, the Netherlands). Cells were grown in DMEM (BioWhittaker Europe, Verviers, Belgium) containing 10% foetal calf serum (FCS) (BioWitthaker), 5 mM L-glutamine, 20,000 units penicillin/streptomycin (BioWhittaker) and 5 mM non-essential amino acids (Gibco, Paisley, United Kingdom). Flasks with cells were incubated at 37°C in 5% CO$_2$ for 3 or 4 days until cells had grown nearly confluent.

HL60 cells were from ATCC and grown in RPMI 1640 medium (BioWhittaker) with 10% FCS, 5mM L-glutamine and 20,000 units penicillin/streptomycin.

HL-60 cells were fluorescently labeled by incubation for 30 min at 37°C with 5 µM calcein-AM (Molecular Probes, Leiden, The Netherlands) in RPMI. These cells (50,000/ well) were added to cultured CHO cells expressing P-selectin (CHO-P cells, cultured in DMEM with 10% FCS, 5 mM non-essential amino acids, 5 mM L-glutamine and 20.000

U penicillin/streptomycin), seeded in 96 well plates in the presence or absence of the P-selectin antagonists (1h, 4°C) (16). After gentle washing with RPMI, CHO-P associated fluorescence was measured (λ_{exc} 485/ λ_{em} 530 nm).

For the results see figure 6.

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